

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problems Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 16/28, 16/18, 14/485, C12N 5/18, C07H 21/00, A61K 49/00, 51/10		A1	(11) International Publication Number: WO 95/21868
			(43) International Publication Date: 17 August 1995 (17.08.95)
(21) International Application Number: PCT/US95/01678			(81) Designated States: AU, CA, CN, FI, HU, JP, KR, NO, RU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(22) International Filing Date: 10 February 1995 (10.02.95)			
(30) Priority Data: 08/196,041 10 February 1994 (10.02.94) US 08/326,552 20 October 1994 (20.10.94) US			
(71) Applicant: IMCLONE SYSTEMS INCORPORATED [US/US]; 180 Varick Street, New York, NY 10014 (US).			
(72) Inventors: ROCKWELL, Patricia; 69 Old Stagecoach Road, West Redding, CT 06896 (US). GOLDSTEIN, Neil, L; 26 Kendal Avenue, Maplewood, NJ 07040 (US).			
(74) Agents: FEIT, Irving, N. et al.; ImClone Systems Incorporated, 180 Varick Street, New York, NY 10014 (US).			Published With international search report.
(54) Title: MONOCLONAL ANTIBODIES SPECIFIC TO VEGF RECEPTORS AND USES THEREOF			
(57) Abstract Monoclonal antibodies that specifically bind to an extracellular domain of a VEGF receptor and neutralize activation of the receptor are provided. <i>In vitro</i> and <i>in vivo</i> methods of using these antibodies are also provided.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

**MONOCLONAL ANTIBODIES SPECIFIC TO VEGF RECEPTORS AND USES
THEREOF**

BACKGROUND OF THE INVENTION

5 Angiogenesis is the process of developing new blood vessels that involves the proliferation, migration and tissue infiltration of capillary endothelial cells from pre-existing blood vessels. Angiogenesis is important in normal physiological processes including embryonic development, follicular growth, and wound healing as well as in pathological conditions involving tumor growth and non-neoplastic diseases involving
10 abnormal neovascularization, including neovascular glaucoma (Folkman, J. and Klagsbrun, M. Science **235**:442-447 (1987)).

The vascular endothelium is usually quiescent and its activation is tightly regulated during angiogenesis. Several factors have been implicated as possible regulators of
15 angiogenesis *in vivo*. These include transforming growth factor (TGF β), acidic and basic fibroblast growth factor (aFGF and bFGF), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (Klagsbrun, M. and D'Amore, P. (1991) Annual Rev. Physiol. **53**: 217-239). VEGF, an endothelial cell-specific mitogen, is distinct among these factors in that it acts as an angiogenesis inducer by specifically
20 promoting the proliferation of endothelial cells.

VEGF is a homodimeric glycoprotein consisting of two 23 kD subunits with structural similarity to PDGF. Four different monomeric isoforms of VEGF exist resulting from

5 alternative splicing of mRNA. These include two membrane bound forms (VEGF₂₀₆ and VEGF₁₈₉) and two soluble forms (VEGF₁₆₅ and VEGF₁₂₁). In all human tissues except placenta, VEGF₁₆₅ is the most abundant isoform.

VEGF is expressed in embryonic tissues (Breier et al., Development (Camb.) 114:521
10 (1992)), macrophages, proliferating epidermal keratinocytes during wound healing (Brown et al., J. Exp. Med., 176:1375 (1992)), and may be responsible for tissue edema associated with inflammation (Ferrara et al., Endocr. Rev. 13:18 (1992)). *In situ* hybridization studies have demonstrated high VEGF expression in a number of human tumor lines including glioblastoma multiforme, hemangioblastoma, central nervous
15 system neoplasms and AIDS-associated Kaposi's sarcoma (Plate, K. et al. (1992) Nature 359: 845-848; Plate, K. et al. (1993) Cancer Res. 53: 5822-5827; Berkman, R. et al. (1993) J. Clin. Invest. 91: 153-159; Nakamura, S. et al. (1992) AIDS Weekly, 13 (1)). High levels of VEGF were also observed in hypoxia induced angiogenesis (Shweiki, D. et al. (1992) Nature 359: 843-845).

20

The biological response of VEGF is mediated through its high affinity VEGF receptors which are selectively expressed on endothelial cells during embryogenesis (Millauer, B., et al. (1993) Cell 72: 835-846) and during tumor formation. VEGF receptors typically are class III receptor-type tyrosine kinases characterized by having several, typically 5
25 or 7, immunoglobulin-like loops in their amino-terminal extracellular receptor ligand-binding domains (Kaipainen et al., J. Exp. Med. 178:2077-2088 (1993)). The other two regions include a transmembrane region and a carboxy-terminal intracellular catalytic domain interrupted by an insertion of hydrophilic interkinase sequences of variable

5 lengths, called the kinase insert domain (Terman et al., *Oncogene* 6:1677-1683 (1991)).
VEGF receptors include *flt-1*, sequenced by Shibuya M. et al., *Oncogene* 5, 519-524
(1990); *KDR*, described in PCT/US92/01300, filed February 20, 1992, and in Terman et
al., *Oncogene* 6:1677-1683 (1991); and *flk-1*, sequenced by Matthews W. et al. *Proc.*
Natl. Acad. Sci. USA, 88:9026-9030 (1991).

10

High levels of *flk-1* are expressed by endothelial cells that infiltrate gliomas (Plate, K. et
al., (1992) *Nature* 359: 845-848). *Flk-1* levels are specifically upregulated by VEGF
produced by human glioblastomas (Plate, K. et al. (1993) *Cancer Res.* 53: 5822-5827).

The finding of high levels of *flk-1* expression in glioblastoma associated endothelial
15 cells (GAEC) indicates that receptor activity is probably induced during tumor formation
since *flk-1* transcripts are barely detectable in normal brain endothelial cells. This
upregulation is confined to the vascular endothelial cells in close proximity to the tumor.
Blocking VEGF activity with neutralizing anti-VEGF monoclonal antibodies (mAbs)
resulted in an inhibition of the growth of human tumor xenografts in nude mice (Kim, K.
20 et al. (1993) *Nature* 362: 841-844), indicating a direct role for VEGF in tumor-related
angiogenesis.

Although the VEGF ligand is upregulated in tumor cells, and its receptors are
upregulated in tumor infiltrated vascular endothelial cells, the expression of the VEGF
25 ligand and its receptors is low in normal cells that are not associated with angiogenesis.
Therefore, such normal cells would not be affected by blocking the interaction between
VEGF and its receptors to inhibit angiogenesis, and therefore tumor growth. Blocking

- 5 this VEGF-VEGF receptor interaction by using a monoclonal antibody to the VEGF receptor has not been described prior to the subject invention.

One advantage of blocking the VEGF receptor as opposed to blocking the VEGF ligand to inhibit angiogenesis, and thereby to inhibit pathological conditions such as tumor
10 growth, is that fewer antibodies may be needed to achieve such inhibition. Furthermore, receptor expression levels may be more constant than those of the environmentally induced ligand. Another advantage of blocking the VEGF receptor is that more efficient inhibition may be achieved when combined with blocking of the VEGF ligand.

- 15 The object of this invention is to provide a monoclonal antibody that neutralizes the interaction between VEGF and its receptor by binding to a VEGF receptor and thereby preventing VEGF phosphorylation of the receptor. A further object of this invention is to provide methods to inhibit angiogenesis and thereby to inhibit tumor growth in mammals using such monoclonal antibodies.

20

SUMMARY OF THE INVENTION

- 25 The present invention provides a monoclonal antibody which specifically binds to an extracellular domain of a VEGF receptor and neutralizes activation of the receptor.

- 5 The invention also provides a hybridoma cell line DC101 (IgG1k) producing the monoclonal antibody deposited as ATCC Accession No. ATCC HB 11534, as well as the monoclonal antibody produced therefrom.

- Further, the invention provides a method of neutralizing VEGF activation of a VEGF
10 receptor in endothelial cells comprising contacting the cells with the monoclonal antibody of the invention.

- The invention also provides a method of inhibiting angiogenesis in a mammal comprising administering an effective amount of any one of the antibodies of the
15 invention to the mammal. In addition, the invention provides a method of inhibiting tumor growth in a mammal comprising administering an effective amount of any one of the antibodies of the invention to the mammal.

- The invention also provides a pharmaceutical composition comprising any one of the
20 antibodies of the invention and a pharmaceutically acceptable carrier.

DESCRIPTION OF THE FIGURES

- 25 **Figure 1:** Western Blot of flk-1/SEAPS immunoprecipitation with monoclonal antibody DC101 demonstrating that DC101 immunoprecipitates murine flk-1:SEAPS but not SEAPS alone.

5 **Figure 2a:** Competitive inhibition assay indicating the effect of anti-flk-1 monoclonal antibody DC101 on VEGF₁₆₅ induced phosphorylation of the flk-1/fms receptor in transfected 3T3 cells.

Figure 2b: Sensitivity of VEGF induced phosphorylation of the flk-1/fms receptor to inhibition by monoclonal antibody DC101. C441 cells were assayed at maximal stimulatory concentrations of VEGF₁₆₅ (40 ng/ml) combined with varying levels of the antibody.

Figure 3: Titration of VEGF-induced phosphorylation of the flk-1/fms receptor in the presence of mAb DC101. C441 cells were stimulated with the concentrations of VEGF indicated in the presence (Lanes 1 to 4) or absence (Lanes 5 to 8) of 5 ug/ml of MAb DC101. Unstimulated cells assayed in the presence of antibody (Lane 9) serves as the control. Densitometry scans of the level of phosphorylated receptor in each lane relative to each VEGF concentration is plotted to show the extent of MAb inhibition at excess ligand concentrations. Cell lysates were prepared for detection by anti-phosphotyrosine as described in the Examples below.

Figure 4: Inhibition of VEGF-flk-1/fms activation by prebound mAb DC101. C441 cells were stimulated with the concentrations of VEGF indicated in the absence (Lanes 3 and 4) and presence (Lanes 5 and 6) of DC101. Unstimulated cells (Lanes 1 and 2) serve as controls. MAb was assayed using two sets of conditions. For P, cells were prebound with Mab followed by stimulation with VEGF for 15 minutes at room temperature. For C, Mab and ligand were added simultaneously and assayed as above.

5

Figure 5: VEGF-induced phosphorylation of the flk-1/fms receptor by treatments with varying concentrations of monoclonal antibody DC101 and conditioned media from glioblastoma cells (GB CM).

10 **Figure 6:** FACS analysis of anti-flk-1 mAb binding to flk-1/fms transfected 3T3 Cells (C441). Transfected flk-1/fms 3T3 cells were incubated on ice for 60 minutes with 10 ug/ml of the anti-flk-1 MAb DC101 or the isotype matched irrelevant anti-flk-1 MAb 23H7. Cells were washed and reincubated with 5 ug of goat anti-mouse IgG conjugated to FITC, washed, and analyzed by flow cytometry to determine antibody binding. Data
15 shows the level of fluorescence for DC101 to C441 cells relative to that detected with the irrelevant MAb 23H7.

Figure 7: Saturation binding of mAb DC101 to the flk-1/fms receptor on the transfected 3T3 cell line C441. Confluent C441 cells were incubated in 24 well plates with
20 increasing concentrations of MAb DC101 (50 ng/ml to 2 ug/ml) for two hours at 4°C. Cells were washed and incubated with 5 ug anti-rat IgG-biotin conjugate. To detect binding, cells were washed, incubated with a 1:1000 dilution of streptavidin-HRP, washed and incubated in a colormetric detection system (TMB). Data represents the
25 absorbance at 540 nm versus increasing concentrations of MAb DC101. The binding of the secondary antibody to cells alone was subtracted from each determination to adjust for non-specific binding. Data represents the average of three independent experiments.

5 **Figure 8:** Immunoprecipitation of phosphorylated flk-1/fms from VEGF stimulated flk-1/fms transfected 3T3 cells. Cells were stimulated with VEGF as described in the Experimental Procedures and lysates were immunoprecipitated with irrelevant or relevant antibodies as follows: 1. rat anti-flk2 IgG2a (Mab 2A13); 2. rat anti-flk-1 IgG1 (Mab DC101); 3. rat anti-flk2 IgG1 (Mab 23H7); 4. rabbit anti-fms polyclonal antibody.

10 Immunoprecipitated protein was subjected to SDS PAGE followed by Western blotting. The immunoprecipitation of VEGF activated receptor was detected by probing the blots with an anti-phosphotyrosine antibody.

Figure 9: Sensitivity of VEGF-induced phosphorylation of the flk-1/fms receptor to inhibition by mAb DC101. Prebound and competitive assays were performed with 40 ng/ml of VEGF at the antibody concentrations indicated. Cell lysates were prepared for receptor detection with anti-phosphotyrosine as described in the Examples below.

Figure 10: Effect of mAb DC101 on CSF-1 induced phosphorylation of the FMS receptor. In (B), the fms/flk-2 transfected 3T3 cell line, 10A2, was stimulated with optimal stimulatory levels of CSF-1 in the absence (Lanes 3 and 4) and presence (Lanes 5 and 6) of 5 ug/ml of MAb DC101. Unstimulated cells assayed in the absence (Lane 1) or presence (Lane 2) of antibody serve as controls. Cell lysates were prepared for detection by anti-phosphotyrosine as described in the Examples below.

25

Figure 11: Specificity of mAb DC101 neutralization of the activated flk-1/fms receptor. C441 cells were stimulated with 20 or 40 ng/ml of VEGF in the presence of DC101 (IgG1) or the irrelevant anti-flk-2 rat monoclonal antibodies 2A13 (IgG2a) or 23H7

5 (IgG1). Assays were performed with each antibody in the absence of VEGF (Lanes 1 to 3) and in the presence of VEGF under competitive (lanes 4 to 8) or prebound (lanes 9 to 11) conditions. Cell lysates were prepared for detection by anti-phosphotyrosine as described in the Examples below. Blots were stripped and reprobed to detect the flk-1/fms receptor using a rabbit polyclonal antibody to the C-terminal region of the fms
10 receptor.

Figure 12: Binding of MAb DC101 to Intact HUVEC Cells. Confluent HUVEC cells were assayed for binding on gelatin coated 24 well microtiter plates with the antibody concentrations as indicated. Binding was determined using the same protocol as that
15 described in the legend to Figure 7.

Figure 13: Immunoprecipitation of phosphorylated receptor bands from VEGF stimulated HUVEC cells. HUVEC cells were grown to subconfluency in endothelial growth medium (EGM) for three days without a change of medium. Receptor forms
20 were immunoprecipitated by MAb DC101 from lysates of unstimulated cells (Lane 1), VEGF stimulated cells (lane 2), and cells stimulated with VEGF in the presence of 1 ug/ml heparin (Lane 3). Phosphorylation assays, immunoprecipitations, and detection of the phosphorylated receptor forms were performed as described in the Experimental Procedures.

25

Figure 14: Effect of mAb DC101 on the proliferation of HUVEC cells in response to VEGF. Cells were grown for 48 hours as described in the legend to Figure 6. Cells were then subjected to the following assay conditions: no addition to medium

- 5 (untreated); a change of fresh endothelial growth medium (complete medium); the addition of 10 ng/ml of VEGF in the absence or presence of 1 ug/ml heparin; and VEGF and VEGF-heparin treated cells assayed in the presence of 1 ug/ml of DC101. Cells were assayed for proliferation by colorimetric detection at 550 nm using a cell proliferation assay kit (Promega).

10

Figure 15: Effect of mAb DC101 on VEGF receptor forms in the tumor cell lines A431 and 8161.

- Figure 16:** Comparison between the control 2A13 group (rat anti-flk-2 monoclonal antibody) and DC101 (rat anti-flk-1 monoclonal antibody) of reduction in tumor growth in individual animals.

- Figure 17:** Athymic nude mice were injected subcutaneously with human glioblastoma cell line GBM-18 and divided into three groups: a PBS control, an irrelevant rat IgG1 control 23H7, and DC101. Treatments were administered simultaneously with tumor xenografts and continued for four weeks.

DETAILED DESCRIPTION OF THE INVENTION

25

The present invention provides monoclonal antibodies that bind specifically to an extracellular domain of a VEGF receptor. An extracellular domain of a VEGF receptor is herein defined as a ligand-binding domain on the amino-terminal, extracellular region

5 of the receptor, typical of class III tyrosine kinase receptors. Some examples of VEGF receptors include the protein tyrosine kinase receptors fit-1, KDR and flk-1. The VEGF receptor is preferably bound to a cell, such as an endothelial cell. The VEGF receptor may also be bound to a non-endothelial cell, preferably a tumor cell. Alternatively, the VEGF receptor may be free from the cell, preferably in soluble form.

10

The antibodies of the invention neutralize activation of the VEGF receptor by preventing binding of the VEGF ligand to the VEGF receptor's extracellular binding domain, and thereby preventing phosphorylation of the receptor.

15

UTILITY

A. Neutralizing VEGF activation of VEGF receptor

20 *In Vivo:*

Neutralization of VEGF activation of a VEGF receptor in a sample of endothelial or non-endothelial cells, such as tumor cells, may be performed *in vivo*, wherein an antibody of the invention is, by administration to a mammal, contacted with a VEGF receptor.

25

Methods of administration to a mammal include, for example, oral, intravenous, intraperitoneal, subcutaneous, or intramuscular administration.

5 This *in vivo* neutralization method is useful for inhibiting angiogenesis in a mammal.

The *in vivo* neutralization method is a useful therapeutic method, such as for preventing or inhibiting angiogenesis associated with pathological conditions such as tumor growth in a mammal. Accordingly, the antibodies of the invention are anti-angiogenic immunotherapeutic agents.

10

Flk-1 receptors were unexpectedly found on non-endothelial cells, such as tumor cells, indicating the unexpected presence of an autocrine loop in these cells. The antibodies of this invention are useful in neutralizing VEGF phosphorylation of these receptors, thereby blocking the autocrine loop and inhibiting tumor growth.

15

The methods of inhibiting angiogenesis and of inhibiting pathological conditions such as tumor growth in a mammal comprises administering an effective amount of any one of the invention's antibodies to a mammal or directly to a tumor within the mammal. The mammal is preferably human. This method is effective for treating subjects with

20 carcinomas or sarcomas, preferably highly vascular tumors such as Kaposi's sarcoma, CNS neoplasms (capillary hemangioblastomas, meningiomas and cerebral metastases), melanoma, gastrointestinal and renal sarcomas, rhabdomyosarcoma, glioblastoma, preferably glioblastoma multiforme, and leiomyosarcoma.

25 A cocktail of approximately 3-6 monoclonal antibodies of the invention provides an especially efficient treatment for inhibiting the growth of tumor cells.

- 5 The combined treatment of one or more of the antibodies of the invention with anti-VEGF antibodies provides a more efficient treatment for inhibiting the growth of tumor cells than the use of the antibody or antibodies alone.

Furthermore, the combined treatment of one or more of the antibodies of the invention
10 with an anti-neoplastic or anti-chemotherapeutic drug such as doxorubicin, cisplatin or taxol provides a more efficient treatment for inhibiting the growth of tumor cells than the use of the antibody by itself. In one embodiment, the pharmaceutical composition comprises the antibody and carrier with an anti-chemotherapeutic drug attached thereto.

- 15 Preventing or inhibiting angiogenesis is also useful to treat non-neoplastic angiogenic pathologic conditions such as neovascular glaucoma, proliferative retinopathy including proliferative diabetic retinopathy, macular degeneration, hemangiomas, angiofibromas, and psoriasis.

20 ***In Vitro:***

The invention provides a method of neutralizing VEGF activation by phosphorylation of a VEGF receptor in a sample of endothelial cells comprising contacting the sample with an antibody of the invention before, simultaneously with, or after, adding VEGF to the
25 cell sample.

Since the flk-1 receptor forms are shown by this invention to be expressed in non-endothelial cells, such as tumor cells, as well as endothelial cells, the invention provides

- 5 a method of neutralizing VEGF receptor activation in non-endothelial cells, preferably tumor cells, comprising contacting the cells with an antibody of the invention before, simultaneously with, or after, adding VEGF to the cells.

10 **B. Using the Antibodies of the Invention to Isolate and Purify the VEGF Receptor**

The antibodies of the present invention may be used to isolate and purify the VEGF receptor using conventional methods such as affinity chromatography (Dean, P.D.G. et al., Affinity Chromatography: A Practical Approach, Arlington, VA:IRL Press (1985)).

- 15 Other methods well known in the art include magnetic separation with antibody-coated magnetic beads, "panning" with an antibody attached to a solid matrix, and flow cytometry.

- The source of VEGF receptor is typically vascular endothelial cells that express the
20 VEGF receptor. Suitable sources of vascular endothelial cells are blood vessels.

The VEGF receptors may be used as starting material to produce other materials, such as DNA encoding the receptors, or as antigen for making additional monoclonal and polyclonal antibodies that recognize and bind to the VEGF receptor or other antigens on the surface of VEGF-expressing cells.

25

C. Using the Antibodies of the Invention to Isolate and Purify Flk-1 Positive Tumor Cells

- 5 The antibodies of the present invention may be used to isolate and purify flk-1 positive tumor cells, i.e., tumor cells expressing the flk-1 receptor, using conventional methods such as affinity chromatography (Dean, P.D.G. et al., Affinity Chromatography: A Practical Approach, Arlington, VA: IRL Press (1985)). Other methods well known in the art include magnetic separation with antibody-coated magnetic beads, cytotoxic agents, 10 such as complement, conjugated to the antibody, "panning" with an antibody attached to a solid matrix, and flow cytometry.

D. Monitoring Levels of VEGF *In Vitro* or *In Vivo*

- 15 The antibodies of the invention may be used to monitor levels of VEGF *in vitro* or *in vivo* in biological samples using standard assays and methods known in the art. Some examples of biological samples include bodily fluids, such as blood. Standard assays involve, for example, labelling the antibodies and conducting standard immunoassays, such as radioimmunoassays, as is well known in the art.

20

PREPARATION OF ANTIBODIES

- The monoclonal antibodies of the invention that specifically bind to the VEGF receptor may be produced by methods known in the art. These methods include the 25 immunological method described by Kohler and Milstein in Nature 256, 495-497 (1975) and Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al., Eds., Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science

- 5 Publishers, Amsterdam (1985); as well as by the recombinant DNA method described by Huse et al in Science 246, 1275-1281 (1989).

The antibody may be prepared in any mammal, including mice, rats, rabbits, goats and humans. The antibody may be a member of one of the following immunoglobulin
10 classes: IgG, IgM, IgA, IgD, or IgE, and the subclasses thereof, and preferably is an IgG1 antibody.

In one embodiment the antibody is a monoclonal antibody directed to an epitope of a VEGF receptor present on the surface of a cell. In another embodiment the monoclonal
15 antibody is a rat IgG1 monoclonal antibody, specific for the murine VEGF receptor flk-1, and produced by hybridoma DC101. Hybridoma cell line DC101 was deposited January 26, 1994 with the American Type Culture Collection, designated ATCC HB 11534. In a preferred embodiment, the monoclonal antibody is directed to an epitope of a human flt-1 receptor or to a human KDR receptor.

20

Functional Equivalents of Antibodies

The invention also includes functional equivalents of the antibodies described in this
25 specification. Functional equivalents have binding characteristics comparable to those of the antibodies, and include, for example, chimerized, humanized and single chain antibodies as well as fragments thereof. Methods of producing such functional equivalents are disclosed in PCT Application No. WO 93/21319, European Patent

- 5 Application No. EPO 239,400; PCT Application Wo 89/09622; European Patent Application No. 338,745; and European Patent Application EPO 332,424.

Functional equivalents include polypeptides with amino acid sequences substantially the same as the amino acid sequence of the variable or hypervariable regions of the antibodies of the invention. "Substantially the same" amino acid sequence is defined herein as a sequence with at least 70% percent homology to an amino acid sequence of an antibody of the invention, as determined by the FASTA search method in accordance with Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85, 2444-2448 (1988).

- 15 Chimerized antibodies preferably have constant regions derived substantially or exclusively from human antibody constant regions and variable regions derived substantially or exclusively from the sequence of the variable region from a mammal other than a human.

Humanized antibodies preferably have constant regions and variable regions other than the complement determining regions (CDRs) derived substantially or exclusively from the corresponding human antibody regions and CDRs derived substantially or exclusively from a mammal other than a human.

Suitable mammals other than a human include any mammal from which monoclonal antibodies may be made, such as a rabbit, rat, mouse, horse, goat, or primate.

Single chain antibodies or Fv fragments are polypeptides which consist of the V region of the heavy chain of the antibody linked to the V region of the light chain with or without

- 5 an interconnecting linker. This comprises the entire antibody combining site, and is the minimal antibody binding site. These chains may be produced in bacteria.

Functional equivalents further include fragments of antibodies that have the same or binding characteristics comparable to those of the whole antibody. Such fragments may
10 contain one or both Fab fragments or the F(ab')₂ fragment. Preferably the antibody fragments contain all six complement determining regions of the whole antibody, although fragments containing fewer than all of such regions, such as three, four or five CDRs, may also be functional.

- 15 Further, the functional equivalents may be or may combine members of any one of the following immunoglobulin classes: IgG, IgM, IgA, IgD, or IgE, and the subclasses thereof.

Preparation of VEGF Receptor Immunogens

20

The VEGF receptor may be used as an immunogen to raise an antibody of the invention. Alternatively, synthetic VEGF receptor peptides may be prepared using commercially available machines and the VEGF receptor amino acid sequence provided by, for example, Shibuya M. et al., Oncogene 5, 519-524 (1990) for flt-1;

- 25 PCT/US92/01300 and Terman et al., Oncogene 6:1677-1683 (1991) for KDR; and Matthews W. et al. Proc. Natl. Acad. Sci. USA, 88:9026-9030 (1991) for flk-1.

5 As a further alternative, DNA encoding a VEGF receptor, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen to raise an antibody of the invention. In order to prepare the VEGF receptors against which the antibodies are made, nucleic acid molecules that encode the VEGF receptors of the invention, or portions thereof, especially the
10 extracellular portions thereof, may be inserted into known vectors for expression in host cells using standard recombinant DNA techniques. Standard recombinant DNA techniques are described in Sambrook et al., "Molecular Cloning," Second Edition, Cold Spring Harbor Laboratory Press (1987) and by Ausubel et al. (Eds) "Current Protocols in Molecular Biology," Green Publishing Associates/ Wiley-Interscience, New York (1990).

15

A suitable source of cells containing nucleic acid molecules that express the VEGF receptor includes vascular endothelial cells.

20 Total RNA is prepared by standard procedures from endothelial receptor-containing tissue. The total RNA is used to direct cDNA synthesis. Standard methods for isolating RNA and synthesizing cDNA are provided in standard manuals of molecular biology such as, for example, in Sambrook et al., "Molecular Cloning," Second Edition, Cold Spring Harbor Laboratory Press (1987) and in Ausubel et al., (Eds), "Current Protocols
25 in Molecular Biology," Greene Associates/Wiley Interscience, New York (1990).

The cDNA of the receptors may be amplified by known methods. For example, the cDNA may be used as a template for amplification by polymerase chain reaction (PCR);

5 see Saiki et al., Science, 239, 487 (1988) or Mullis et al., U.S. patent 4,683,195. The sequences of the oligonucleotide primers for the PCR amplification are derived from the sequences of mouse and human VEGF receptor respectively. The oligonucleotides are synthesized by methods known in the art. Suitable methods include those described by Caruthers in Science 230, 281-285 (1985).

10

In order to isolate the entire protein-coding regions for the VEGF receptors, the upstream PCR oligonucleotide primer is complementary to the sequence at the 5' end, preferably encompassing the ATG start codon and at least 5-10 nucleotides upstream of the start codon. The downstream PCR oligonucleotide primer is complementary to the
15 sequence at the 3' end of the desired DNA sequence. The desired DNA sequence preferably encodes the entire extracellular portion of the VEGF receptor, and optionally encodes all or part of the transmembrane region, and/or all or part of the intracellular region, including the stop codon. A mixture of upstream and downstream oligonucleotides are used in the PCR amplification. The conditions are optimized for
20 each particular primer pair according to standard procedures. The PCR product is analyzed by electrophoresis for cDNA having the correct size, corresponding to the sequence between the primers.

Alternatively, the coding region may be amplified in two or more overlapping fragments.

25 The overlapping fragments are designed to include a restriction site permitting the assembly of the intact cDNA from the fragments.

- 5 The DNA encoding the VEGF receptors may also be replicated in a wide variety of cloning vectors in a wide variety of host cells. The host cell may be prokaryotic or eukaryotic.

The vector into which the DNA is spliced may comprise segments of chromosomal, non-
10 chromosomal and synthetic DNA sequences. Some suitable prokaryotic cloning vectors include plasmids from E. coli, such as colE1, pCR1, pBR322, pMB9, pUC, pKSM, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as M13 and other filamentous single-stranded DNA phages.

- 15 A preferred vector for cloning nucleic acid encoding the VEGF receptor is the Baculovirus vector.

Expression and Isolation of Receptor Immunogens

- 20 DNA encoding the VEGF receptors of the invention are inserted into a suitable expression vector and expressed in a suitable prokaryotic or eucaryotic host. The DNA inserted into a host may encode the entire extracellular portion of the VEGF receptor, or a soluble fragment of the extracellular portion of the VEGF receptor. The extracellular portion of the VEGF receptor encoded by the DNA is optionally attached at either, or
25 both, the 5' end or the 3' end to additional amino acid sequences. The additional amino acid sequence may be attached to the VEGF receptor extracellular region in nature, such as the leader sequence, the transmembrane region and/or the intracellular region of the VEGF receptor. The additional amino acid sequences may also be sequences

- 5 not attached to the VEGF receptor in nature. Preferably, such additional amino acid sequences serve a particular purpose, such as to improve expression levels, secretion, solubility, or immunogenicity.

Vectors for expressing proteins in bacteria, especially *E. coli*, are known. Such vectors
10 include the PATH vectors described by Dieckmann and Tzagoloff in J. Biol. Chem. 260, 1513-1520 (1985). These vectors contain DNA sequences that encode anthranilate synthetase (TrpE) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (pEX); lambda P_L; maltose binding protein (pMAL); and glutathione S-transferase (pGST) -see Gene 67, 31 (1988) and
15 Peptide Research 3, 167 (1990).

Vectors useful in yeast are available. A suitable example is the 2μ plasmid.

Suitable vectors for use in mammalian cells are also known. Such vectors include well-
20 known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences and shuttle vectors derived from combination of functional mammalian vectors, such as those described above, and functional plasmids and phage DNA.

Further eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P.
25 Berg, J. Mol. Appl. Genet. 1, 327-341 (1982); S. Subramani et al, Mol. Cell. Biol. 1, 854-864 (1981); R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene," J. Mol. Biol. 159, 601-621 (1982); R.J. Kaufmann and P.A. Sharp, Mol.

- 5 Cell. Biol. 159, 601-664 (1982); S.I. Scahill et al, "Expression And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," Proc. Natl. Acad. Sci. USA 80, 4654-4659 (1983); G. Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci. USA 77, 4216-4220, (1980).
- 10 The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system,
- 15 major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, and other sequences known to
- 20 control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Vectors containing the receptor-encoding DNA and control signals are inserted into a host cell for expression of the receptor. Some useful expression host cells include well-

25 known prokaryotic and eukaryotic cells. Some suitable prokaryotic hosts include, for example, E. coli, such as E. coli SG-936, E. coli HB 101, E. coli W3110, E. coli X1776, E. coli X2282, E. coli DHI, and E. coli MRCI, Pseudomonas, Bacillus, such as Bacillus subtilis, and Streptomyces. Suitable eukaryotic cells include yeast and other fungi,

- 5 insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

- Following expression in a host cell maintained in a suitable medium, the VEGF receptors may be isolated from the medium, and purified by methods known in the art. If
- 10 the VEGF receptors are not secreted into the culture medium, the host cells are lysed prior to isolation and purification.

- The antibodies of the invention may also be prepared from VEGF receptors bound to the surface of cells that express the VEGF receptor. The cell to which the VEGF
- 15 receptors are bound may be a cell that naturally expresses the receptor, such as a vascular endothelial cell. Alternatively, the cell to which the receptor is bound may be a cell into which the DNA encoding the receptor has been transfected, such as 3T3 cells.

20

EXAMPLES:

- 25 The Examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be construed to, limit its scope in any way. The Examples do not include detailed descriptions of conventional methods employed in the construction of vectors and plasmids, the insertion of genes encoding polypeptides into

5 such vectors and plasmids or the introduction of plasmids into hosts. Such methods are well known to those of ordinary skill in the art and are described in numerous publications including Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press.

10 Experimental results of this invention show that DC101 specifically blocks VEGF₁₆₅ induced phosphorylation of a mouse flk-1/fms chimeric receptor expressed in transfected 3T3 cells. The Mab had no effect on a fully stimulated chimeric fms/flk2 receptor by CSF-1. *In vivo* studies described below show that the mAb was able to significantly inhibit tumor growth in nude mice.

15

IN VITRO STUDIES USING DC101

Experimental Procedures

20 Cell Lines and Media

NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville MD).

The C441 cell line was constructed by transfecting 3T3 cells with the chimeric receptor mouse flk1/human fms. 10A2 is a 3T3 transfectant containing the chimeric receptor

25 human fms/mouse flk2, the isolation and characterization of which has been described (Dosil, M. et al., Mol. Cell. Biol. 13:6572-6585 (1993)). Cells were routinely maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (CS), 1 mM L-glutamine, antibiotics, and 600 ug/ml G418 (Geneticin; Sigma, St Louis MO).

5

A glioblastoma cell line was maintained in DME supplemented with 5% CS, 1mM L-glutamine, and antibiotics.

A stable 3T3 line secreting the soluble chimeric protein, mouse flk1:SEAPs (secretory alkaline phosphatase) was generated and maintained.

Isolation of Monoclonal Antibodies

Lewis rats (Charles River Labs) were hyperimmunized with an immune complex consisting of the mouse flk-1:SEAPs soluble receptor immunoprecipitated using a rabbit anti-alkaline phosphatase polyclonal antibody and Protein-G sepharose beads. The animals received 7 intraperitoneal injections of this complex spread over 3 months (days 0, 14, 21, 28, 49, 63, 77). At various times, the animals were bled from the tail vein and immune sera screened by ELISA for high titer binding to mflk-1:SEAPs. Five days after the final injection, rats were sacrificed and the spleens aseptically removed.

Splenocytes were washed, counted, and fused at a 2:1 ratio with the murine myeloma cell line NS1. Hybridomas were selected in HAT medium and colonies screened by ELISA for specific binding to mflk-1:SEAPs but not the SEAPs protein. A number of positive hybridomas were expanded and cloned three times by limiting dilution. One subclone, designated DC101, was further characterized.

5 ELISA Methods

Antibodies were screened by a solid state ELISA in which the binding characteristics of the various mAbs to flk-1:SEAP and SEAP protein were compared. Microtiter plates were coated with 50-100 ng/well of either flk-1:SEAP or AP in pH9.6 carbonate buffer
10 overnight at 4°C. Plates were blocked with phosphate buffered saline supplemented with 10% new born calf serum (NB10) for one hour at 37°C. Hybridoma supernatants or purified antibodies were added to the plates for two hours at 37°C followed by goat anti-rat IgG conjugated to horse radish peroxidase (Tago) added for an additional hour at 37°C. After extensive washing, TMB (Kirkegaard and Perry, Gaithersburg MD) plus
15 hydrogen peroxide was added as the chromogen and the plates read at 450 nm in an ELISA reader.

Isotyping

20 Isotyping of the various monoclonal antibodies was done as previously described (Songsakphisam, R. and Goldstein, N.I., Hybridoma 12: 343-348, 1993) using rat isotype specific reagents (Zymed Labs, South San Francisco CA).

25 Phosphorylation, Immunoprecipitation and Immunoblot Assays

The phosphorylation assays and Western blot analysis with C441 and 10A2 cells were performed as previously described (Tessler et al., 1994) with some modifications.

5 Briefly, cells were grown to 90% confluency in DME-10% CS and then serum starved in DME-0.5% CS for 24 hours prior to experimentation. HUVEC cells were grown to subconfluence in EGM basal media. For neutralization assays, cells were stimulated with various concentrations of the appropriate ligand under serum free conditions (DME-0.1% BSA) in the presence and absence of mAb DC101 for 15 minutes at room
10 temperature. The ligands, VEGF and CSF-1, were assayed at concentrations of 10-80 ng/ml and 20-40 ng/ml, respectively. Monoclonal antibodies were assayed at concentrations ranging from 0.5 ug/ml to 10 ug/ml. To evaluate the effects of mAb DC101 on the VEGF induced activation of the flk-1-fms receptor, antibody was either added simultaneously (competitive inhibition) or prebound to cells for 15 minutes at
15 room temperature prior to the addition of ligand. Cells incubated in serum free medium in the absence and presence of DC101 served as controls for receptor autophosphorylation in the absence of ligand and the presence of antibody, respectively. A control cell line expressing the fms/flk2 chimeric receptor (10A2) was starved and stimulated with 20 and 40 ng/ml CSF-1 and assayed in the presence and
20 absence of 5 ug/ml DC101.

Following stimulation, monolayers were washed with ice cold PBS containing 1mM sodium orthovanadate. Cells were then lysed in lysis buffer [(20 mM Tris-HCl, pH 7.4,
25 1% Triton X-100, 137mM NaCl, 10% glycerol, 10 mM EDTA, 2mM sodium orthovanadate, 100 mM NaF, 100mM sodium pyrophosphate, 5mM Pefabloc (Boehringer Mannheim Biochemicals, Indianapolis IN), 100 ug aprotinin and 100 ug/ml leupeptin] and centrifuged at 14000 x g for 10 minutes. Protein was immunoprecipitated

5 from cleared lysates of transfected cells using polyclonal antibodies generated to peptides corresponding to the C-terminal region of the human fms receptor (Tessler et al., J. Biol. Chem. 269, 12456-12461, 1994) or the murine flk-2 interkinase domain (Small et al., Proc. Natl. Acad. Sci. USA, 91, 459-463, 1994) coupled to Protein A Sepharose beads. Where indicated, immunoprecipitations with DC101 or irrelevant rat
10 IgG were performed with 10 ug of antibody coupled to Protein G beads. The beads were then washed once with 0.2% Triton X-100, 10 mM Tris-HCl pH8.0, 150 mM NaCl, 2mM EDTA (Buffer A), twice with Buffer A containing 500 mM NaCl and twice with Tris-HCl, pH 8.0. Drained beads were mixed with 30 ul in 2x SDS loading buffer and subjected to SDS PAGE in 4-12% gradient gels (Novex, San Diego CA). After
15 electrophoresis, proteins were blotted to nitrocellulose filters for analysis. Filters were blocked overnight in blocking buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl (TBS) containing 5% bovine serum albumin and 10% nonfat dried milk (Biorad, CA). To detect phosphorylated receptor, blots were probed with a monoclonal antibody directed to phosphotyrosine (UBI, Lake Placid, NY) in blocking buffer for 1 hour at room
20 temperature. Blots were then washed extensively with 0.5 x TBS containing 0.1% Tween-20 (TBS-T) and incubated with goat anti-mouse Ig conjugated to horseradish peroxidase (Amersham). Blots were washed with TBS and incubated for 1 minute with a chemiluminescence reagent (ECL, Amersham). Anti-phosphotyrosine reacting with phosphorylated proteins was detected by exposure to a high performance luminescence
25 detection film (Hyperfilm-ECL, Amersham) for 0.5 to 10 minutes.

- 5 To detect flk-1/fms in C441 cells receptor levels, blots were stripped according to manufacturer's protocols (Amersham) and reprobed with the anti-fms rabbit polyclonal antibody.

Flow Cytometer Binding Assays

10

- C441 cells were grown to near confluency in 10 cm plates. Cells were removed with a non-enzymatic dissociation buffer (Sigma), washed in cold serum free medium and resuspended in Hanks balanced salt solution supplemented with 1% BSA (HBSS-BSA) at a concentration of 1 million cells per tube. mAb DC101 or an isotype matched
- 15 irrelevant antibody anti flk-2 23H7 was added at 10 ug per tube for 60 minutes on ice. After washing, 5ul of goat anti-mouse IgG conjugated to FITC (TAGO) was added for an additional 30 minutes on ice. Cells were washed three times, resuspended in 1 ml of HBSS-BSA, and analyzed on a Coulter Epics Elite Cytometer. Non-specific binding of the fluorescent secondary antibody was determined from samples lacking the primary
- 20 antibody.

Binding Assays to Intact Cells

25

Assays with C441 cells were performed with cells grown to confluency in 24 well dishes. HUVEC cells were grown to confluency in 6 well dishes. Monolayers were incubated at 4°C for 2 hours with various amounts of mAb DC101 in binding buffer (DMEM, 50 Mm

5 HEPES pH 7.0, 0.5% bovine serum albumin). Cells were then washed with cold phosphate buffered saline (PBS) and incubated with a secondary anti-rat IgG antibody conjugated with biotin at a final concentration of 2.5 ug/ml. After 1 hour at 4°C cells were washed and incubated with a streptavidin-horse radish peroxidase complex for 30 minutes at 4°C. Following washing, cell-bound antibody was determined by measuring
10 the absorbance at 540 nm obtained with a colormetric detection system (TMB, Kirkegaard and Perry). The OD540 nm of the secondary antibody alone served as the control for non-specific binding.

Cell proliferation assays

15

Mitogenic assays were performed using the Cell Titer 96 Non Radioactive Cell Proliferation Assay Kit (Promega Corp., Madison, WI). In this assay proliferation is measured colormetrically as the value obtained from the reduction of a tetrazolium salt by viable cells to a formazan product. Briefly, HUVEC cells were grown in 24 well
20 gelatin-coated plates in EGM basal media at 1000 cells/well. After a 48-hour incubation various components were added to the wells. VEGF was added at 10 ng/ml to the media in the presence and absence of 1 ug/ml of mAb DC101. Where indicated, heparin (Sigma) was added to a final concentration of 1 ug/ml. Cells were then incubated for an additional 3 days. To measure cell growth, a 20 ul aliquot of tetrazolum
25 dye was added to each well and cells were incubated for 3 hrs at 37°C. Cells were solubilized and the absorbance (OD570) of the formazan product was measured as a quantitation of proliferation.

5 **Results**

ELISA and Immunoprecipitation Results

Rat IgG1 monoclonal antibody DC101 was found to be specific for the murine tyrosine
10 kinase receptor flk-1. ELISA data showed that the antibody bound to purified flk-
1:SEAP but not alkaline phosphatase or other receptor tyrosine kinases such as flk-2.
As seen in Figure 1, DC101 immunoprecipitates murine flk-1:SEAPS but not SEAPS
alone.

15 **DC101 Neutralization of Flk-1 Receptor**

Experiments were then done to determine whether DC101 could neutralize
phosphorylation of flk1 in C441 cells by its cognate ligand, VEGF₁₆₅. In these studies,
monoclonal antibody and VEGF were added simultaneously to monolayers for 15
20 minutes at room temperature. These conditions were designed to determine the
competitive effects (competitive inhibition) of the antibody on receptor/ligand binding.
The results of these assays, shown in Figure 2a, indicate that VEGF₁₆₅ induced
phosphorylation of the flk1/fms receptor was markedly reduced when cells were
assayed in the presence of DC101. In addition, these data suggest that the Mab
25 competes with VEGF₁₆₅ to prevent a full activation of receptor by ligand. To determine
the sensitivity of the VEGF-flk1 interaction to inhibition by DC101, C441 cells were
assayed at maximal stimulatory concentrations of VEGF₁₆₅ (40 ng/ml) combined with
varying levels of the antibody. The results of these Mab titrations are shown in Figure

5 2b. A marked decrease in the phosphorylation of flk1 by VEGF₁₆₅ was observed when DC101 was included at concentrations greater than 0.5 ug/ml. These data show that relatively low concentrations of antibody (<1 ug/ml) are sufficient to inhibit receptor activation by ligand. At 5 ug/ml the antibody is able to neutralize VEGF₁₆₅ stimulation of flk1 in the presence of excess ligand at 80 ng/ml (Figure 3). As a control, the effect of
10 DC101 was tested on the fully stimulated fms/flk2 receptor (10A2 cell line) using CSF-1. Under these conditions, DC101 showed no effect on receptor activation.

Mab Inhibition

15

The extent and specificity of Mab inhibition was further assessed by studies in which DC101 was preincubated with cells before the addition of ligand to allow maximal interaction of antibody with receptor. In these experiments, monolayers were incubated with 5 ug/ml of DC101, a rat anti-flk2 Mab (2A13) prepared by conventional techniques
20 (ImClone, NY), and control rat IgG1 (Zymed Labs) for 15 minutes at room temperature prior to the addition of 40 ng/ml of VEGF₁₆₅ for an additional 15 minutes. For comparison, assays were run in which DC101 and VEGF₁₆₅ were added simultaneously (competitive inhibition). The results of these studies (Figure 4) show that preincubation of the anti-flk-1 monoclonal antibody with flk1/fms transfected cells completely abrogates
25 receptor activation by VEGF₁₆₅. Similar results were observed using VEGF₁₂₁ for stimulation. While phosphorylation of flk1 by VEGF is not affected by the addition of irrelevant isotype matched rat antibodies, the reactivity of the same blot probed with the anti-fms polyclonal antibody shows an equal level of receptor protein per lane. These

- 5 data indicate that the inhibition of phosphorylation observed with DC101 was due to the blockage of receptor activation rather than a lack of receptor protein in the test samples.

FACS analysis:

- The mAb was assayed by FACS analysis for binding to 3T3 cells transfected with the
10 flk-1/fms receptor (C441 cells). The results, shown in Figure 6, demonstrate that the chimeric flk-1/fms expressed on the surface of C441 cells is specifically recognized by mAb DC101 and not by an antibody of the same isotype raised against the related tyrosine kinase receptor, flk-2. The efficacy of the mAb-receptor interaction at the cell surface was determined from assays in which varying levels of mAb binding was
15 measured on intact C441 cells. These results, shown in Figure 7, indicate that mAb binds to the flk-1/fms receptor with a relative apparent affinity of approximately 500 ng/ml. These results indicate that the mAb has a strong affinity for cell surface expressed flk-1.

20

Immunoprecipitation:

- The extent of mAb reactivity with the flk-1/fms receptor was further assessed by determining the capacity of the antibody to immunoprecipitate the receptor following
25 activation by VEGF. Figure 8 shows an immunoprecipitation by mAb DC101 of the phosphorylated flk-1/fms receptor from VEGF stimulated C441 cells. The results show that the DC101 monoclonal and anti-fms polyclonal antibodies display similar levels of receptor interaction while rat anti flk-2 antibodies 2H37 (IgG1) and 2A13 (IgG2a) show

5 no reactivity. Experiments were then performed to determine whether mAb DC101 could neutralize the VEGF induced phosphorylation of flk-1/fms at maximal stimulatory concentrations of ligand (40 ng/ml). In these studies, monoclonal antibody was added to monolayers either simultaneously with ligand or prior to ligand stimulation and assayed for 15 minutes at room temperature. These conditions were studied to

10 determine both the competitive effects (competitive inhibition) of the antibody on receptor/ligand binding as well as the efficacy of prebound antibody to prevent receptor activation. The results of these assays, shown in Figure 4, indicate that phosphorylation of the flk-1/fms is reduced by the simultaneous addition of mAb with VEGF and markedly inhibited by antibody prebound to the receptor. A densitometry scan of these

15 data revealed that mAb DC101 interacts with flk-1/fms to inhibit phosphorylation to a level that is 6% (lane 5, P) and 40% (lane 6,C) of the fully stimulated receptor control (lane 4). From these data we infer that mAb DC101 strongly competes with the ligand-receptor interaction to neutralize flk-1 receptor activation. To determine the sensitivity of the VEGF-flk-1 interaction to inhibition by mAb DC101, C441 cells were assayed with

20 maximal VEGF levels in the presence of increasing concentrations of antibody. Assays were performed with the mAb under competitive and prebinding conditions. The results of these mAb titrations are shown in Figure 9. A marked decrease in the phosphorylation of flk-1 is observed when mAb DC101 competes with VEGF antibody at concentrations greater than 0.5 ug/ml. These data also show that relatively low

25 concentrations of prebound antibody (<1 ug/ml) are sufficient to completely inhibit receptor activation by ligand.

5 **Phosphorylation:**

To further evaluate the antagonistic behavior of mAb DC101 on receptor activation, phosphorylation assays were performed in which a fixed amount of antibody (5 ug/ml) was added to C441 cells stimulated with increasing amounts of ligand (Figure 3). The level of phosphorylation induced by each ligand concentration in the presence and
10 absence of mAb DC101 was also quantitated by densitometry readings. The plot of these data given in Figure 3 indicates that the antibody was able to partially neutralize receptor phosphorylation even in the presence of excess amounts of VEGF. To evaluate the specificity of mAb DC101 on receptor activation, the antibody was tested for its ability to competitively inhibit CSF-1 induced activation of the *fms/flk-2* receptor in
15 the 3T3 transfected cell line, 10A2. In these experiments 5 ug/ml of mAb DC101 was tested together with CSF-1 concentrations (20-40 ng/ml) that are known to result in full activation of the receptor. These results, which are shown in Figure 10, indicate that mAb DC101 has no effect on the CSF-1 mediated phosphorylation of the *fms/flk-2* receptor.

20

Preincubation:

The extent and specificity of mAb inhibition was further assessed by studies in which
25 mAb DC101 or irrelevant antibodies were preincubated with cells before the addition of ligand to assure maximal interaction of antibody with receptor. In these experiments, monolayers were preincubated with either 5 ug/ml of DC101, a rat anti-*flk2* mAb (2A13) or a control rat IgG1 (Zymed Labs) prior to the addition of 40 ng/ml of VEGF. For

- 5 comparison, competitive assays were run in which antibodies and VEGF were added simultaneously. The results of these studies show that only the preincubation of the anti-flk-1 monoclonal antibody with flk1/fms transfected cells completely abrogates receptor activation by VEGF while phosphorylation of flk1 by VEGF is not affected by the addition of irrelevant isotype matched rat antibodies. The reactivity of the same blot
- 10 probed with the anti-fms polyclonal (Figure 11) shows an equal level of receptor protein per lane. These data indicate that the lack of phosphorylation observed with mAb DC101 treated cells was due to the blockage of a VEGF-induced phosphorylation of equal amounts of expressed receptor.
- 15 Experiments were then conducted to determine whether the anti flk-1 mAb interacts with homologous receptor forms on human endothelial cells. A titration of increasing concentrations of mAb DC101 on HUVEC cells is shown in Figure 12. The results indicate that the antibody displays a complex binding behavior that results in a pattern with two peaks of activity. The data represent a differential antibody interaction to the
- 20 low and high affinity receptors reported to occur on endothelial cells (Vaisman et al., J. Biol. Chem. 265, 19461-19466, 1990). The specificity of mAb DC101 interaction with VEGF stimulated HUVEC cells was then addressed using phosphorylation assays under similar conditions as those reported for Figure 8. In these studies mAb DC101 immunoprecipitates protein bands from HUVEC cells that have molecular weights
- 25 similar to those reported for crosslinked VEGF-receptor bands when the ligand component is subtracted (Figure 13). These bands display an increased phosphorylation when cells are stimulated by VEGF (compare lanes 1 and 2 in Figure 13). In addition, the VEGF induced phosphorylation of the receptor bands is potentiated

- 5 by the inclusion of 1 ug/ml heparin in the assay (lane 3 in Figure 13). These findings are consistent with previous reports of increased VEGF binding to endothelial cells in the presence of low concentrations of heparin (Gitay-Goren et al., J. Biol. Chem. 267, 6093-6098, 1992).
- 10 An inhibitory effect of mAb on endothelial cells is observed when the antibody was tested in mitogenic assays of HUVEC cells stimulated with VEGF in the presence and absence of antibody (Figure 14). These results show that a marked increase in cell proliferation by VEGF is reduced approximately 35% by mAb DC101. Heparin shows no differential effect on cell growth under the growth conditions employed in these
- 15 assays.

Non-endothelial cells

- 20 Several tumor lines were screened for protein reactivity with DC101 by immunoprecipitation and detection with antiphosphotyrosine. Immunoblots from the cell lines 8161 (melanoma) and A431 (epidermoid carcinoma) yielded phosphorylated bands with molecular weights of 180 and 125 kD. In phosphorylation assays with starved cells, VEGF induced a phosphorylation of these bands that was blocked when
- 25 cells were activated in the presence of mAb DC101 (Figure 15). These results indicate that human flk-1 receptor forms are expressed not only in endothelial cells but in non-endothelial cells as well, such as tumor cells. Since A431 cells secrete high levels of

- 5 VEGF, these results also show the presence of an autocrine loop for VEGF receptor activation.

IN VIVO STUDIES USING DC101

- 10 In vivo studies of this invention were designed to determine if an anti-flk1 monoclonal antibody would block the growth of VEGF- expressing tumor cells. In these experiments, a human glioblastoma multiform cell line was used that has high levels of VEGF message and secretes about 5 ng/ml of VEGF growth factor after a 24 hour conditioning in serum free medium (Figure 5).

15

On day zero, athymic nude mice (nu/nu; Charles River Labs) were injected in the flank with 1-2 million glioblastoma cells. Beginning on the same day, animals received intraperitoneal injections of either DC101 and control antibodies (100 ug/animal). The mice received subsequent antibody treatments on days 3, 5, 7, 10, 12, 14, 17, 19, and

- 20 21. Animals received injections of 100 ug of either DC101 or a control rat antibody to the murine flk2 (2A13) receptor on days 0, 3, 5, 7, 10, 12, 14, 17, 19, and 21 for a total inoculation of 1 mg/animal. Tumors began to appear by day 5 and followed for 50 days. Tumor size was measured daily with a caliper and tumor volume calculated by the following formula: $\frac{\pi}{6} \times \text{larger diameter} \times (\text{smaller diameter})^2$ (Baselga J. Natl. Cancer
25 Inst. 85: 1327-1333). Measurements were taken at least three times per week and tumor volume calculated as described above. One tumor bearing animal in the DC101 group died early in the study and was not used to determine statistical significance between the groups.

5

Figure 16 shows a comparison between the DC101 and the control 2A13 group of reduction in tumor growth over 38 days in individual animals. Although all animals developed tumors of varying sizes and number during the course of the study, DC101-treated mice showed an overall delay in tumor progression. One mouse in the DC101 group remained tumor free until day 49 when a small growth was observed. Even then, tumor growth was markedly suppressed. Statistical analysis of the data was done to assess differences in tumor size between the two groups. Data was subjected to a standard analysis of covariance where tumor size was regressed on time with treatment as a covariate. The results showed that reduction in tumor size over time for the DC101 group was significantly different ($p < 0.0001$) from that seen for 2A13 injected mice.

Figure 17 shows the therapeutic efficacy of DC101 in athymic nude mice transplanted with the human glioblastoma tumor cell line GBM-18, which secretes VEGF. Nude mice were injected subcutaneously with GBM-18 cells and divided into three groups of treatment: a PBS control, an irrelevant rat IgG1 control, and DC101. Treatments were administered simultaneously with tumor xenografts and continued for four weeks. The results showed that GBM-18 tumor growth in DC101 treated nude mice was significantly reduced relative to controls. This experiment indicates that DC101 suppresses tumor growth by blocking VEGF activation of flk-1 on tumor associated vascular endothelial cells, and that DC101 has therapeutic value as an anti-angiogenic reagent against vascularized tumors secreting VEGF.

5 SUPPLEMENTAL ENABLEMENT

The invention as claimed is enabled in accordance with the above specification and readily available references and starting materials. Nevertheless, Applicants have deposited on January 26, 1994 with the American Type Culture Collection, 12301
10 Parklawn Drive, Rockville, Md., 20852 USA (ATCC) the hybridoma cell lines that produce the monoclonal antibodies listed below:

Hybridoma cell line DC101 producing anti-mouse flk-1 monoclonal antibody (ATCC Accession Number ATCC HB 11534).

15

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organism will be made available by ATCC
20 under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

5 **CLAIMS**

What is claimed is:

1. A monoclonal antibody which specifically binds to an extracellular domain of a
10 VEGF receptor and neutralizes activation of the receptor.
2. A purified antibody of claim 1, wherein the VEGF receptor is mammalian.
3. A purified antibody of claim 1, wherein the VEGF receptor is human.
15
4. A purified antibody of claim 3, wherein the human receptor is encoded by the *flt-1*
or *KDR* gene.
5. A monoclonal antibody of claim 1 produced by hybridoma cell line DC101
20 deposited as ATCC Accession No. HB 11534.
6. A hybridoma cell line producing a monoclonal antibody of claim 1.
7. A hybridoma cell line producing a purified antibody of claim 3.
25
8. A hybridoma cell line DC101 producing the monoclonal antibody of claim 5
deposited as ATCC Accession No. ATCC HB 11534.

- 5 9. A polypeptide which comprises an amino acid sequence which is substantially
the same as the amino acid sequence of the variable region of the monoclonal
antibody of claim 1 or claim 5.
10. A nucleic acid that encodes the polypeptide of claim 9.
- 10
11. A chimeric antibody or a fragment thereof comprising the polypeptide of claim 9.
12. A chimeric antibody of claim 11 comprising an amino acid sequence of a human
antibody constant region and an amino acid sequence of a non-human antibody
15 variable region.
13. A chimeric antibody of claim 12, wherein the non-human variable region is
murine.
- 20 14. A polypeptide which comprises an amino acid sequence which is substantially
the same as the amino acid sequence of the hypervariable region of the
monoclonal antibody of claim 1 or claim 5.
15. A nucleic acid that encodes the polypeptide of claim 14.
- 25
16. A humanized antibody or a fragment thereof comprising the polypeptide of claim
14.

- 5 17. The humanized antibody of claim 16 comprising amino acid sequences of
framework and constant regions from a human antibody, and an amino acid
sequence of a non-human antibody hypervariable region.
- 10 18. The humanized antibody of claim 17, wherein the amino acid sequence of the
hypervariable region is murine.
- 15 19. A method of neutralizing VEGF activation of a VEGF receptor in cells comprising
contacting the cells with the monoclonal antibody of any of claims 1, 5, 11 and
16.
- 20 20. A method of claim 19, wherein the cells are endothelial cells.
21. A method of claim 19, wherein the cells are tumor cells.
- 25 22. A method of inhibiting angiogenesis in a mammal comprising administering an
effective amount of any one of the antibodies of claims 1, 5, 11 and 16 to the
mammal.
23. A method of claim 22, wherein the mammal is a human.
24. A method of inhibiting tumor growth in a mammal comprising administering an
effective amount of any one of the antibodies of claims 1, 5, 11 and 16 to the
mammal.

5.

25. A method of claim 24, wherein the mammal is a human.

26. A method of inhibiting tumor growth in a mammal comprising administering an effective amount of any one of the antibodies of claims 1, 5, 11 and 16 and a
10 chemotherapeutic agent.

27. A method of claim 26, wherein the chemotherapeutic agent is selected from the group consisting of doxorubicin, cisplatin and taxol.

15 28. A pharmaceutical composition comprising any one of the antibodies of claims 1, 5, 11 and 16 and a pharmaceutically acceptable carrier.

29. A pharmaceutical composition of claim 27 further comprising a chemotherapeutic agent.

Figure 1

**Western Blot of Flk-1/SEAPS Immunoprecipitation
with MAb DC101**

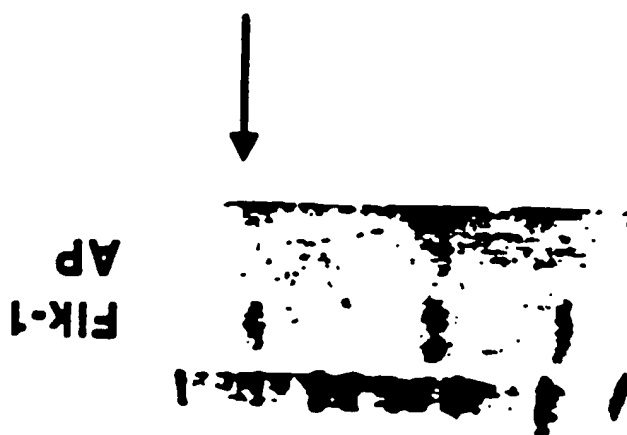


Figure 2a

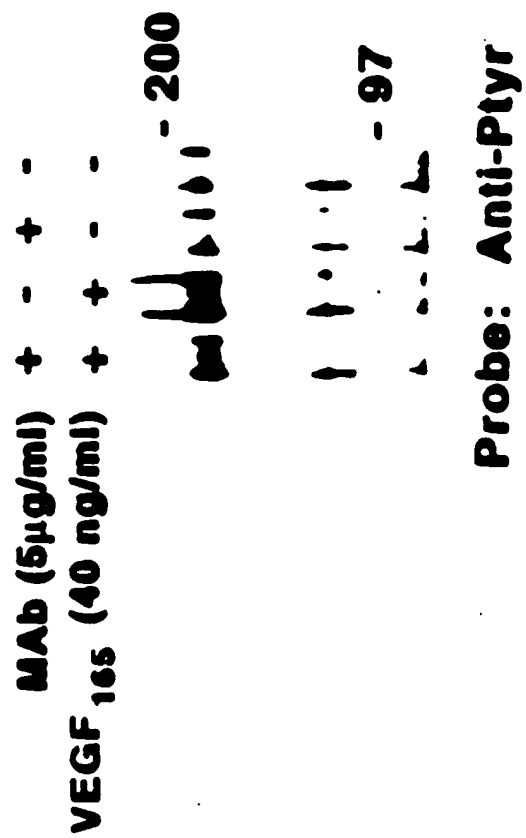


Figure 2b

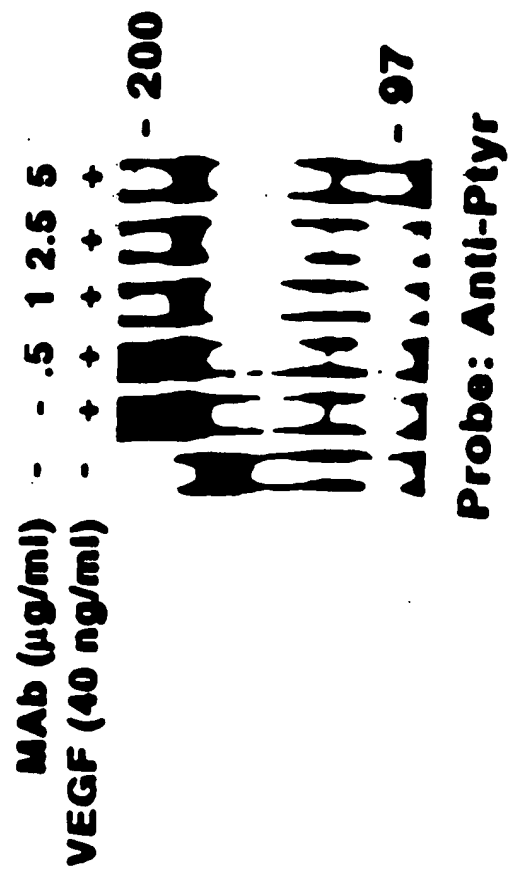


Figure 3

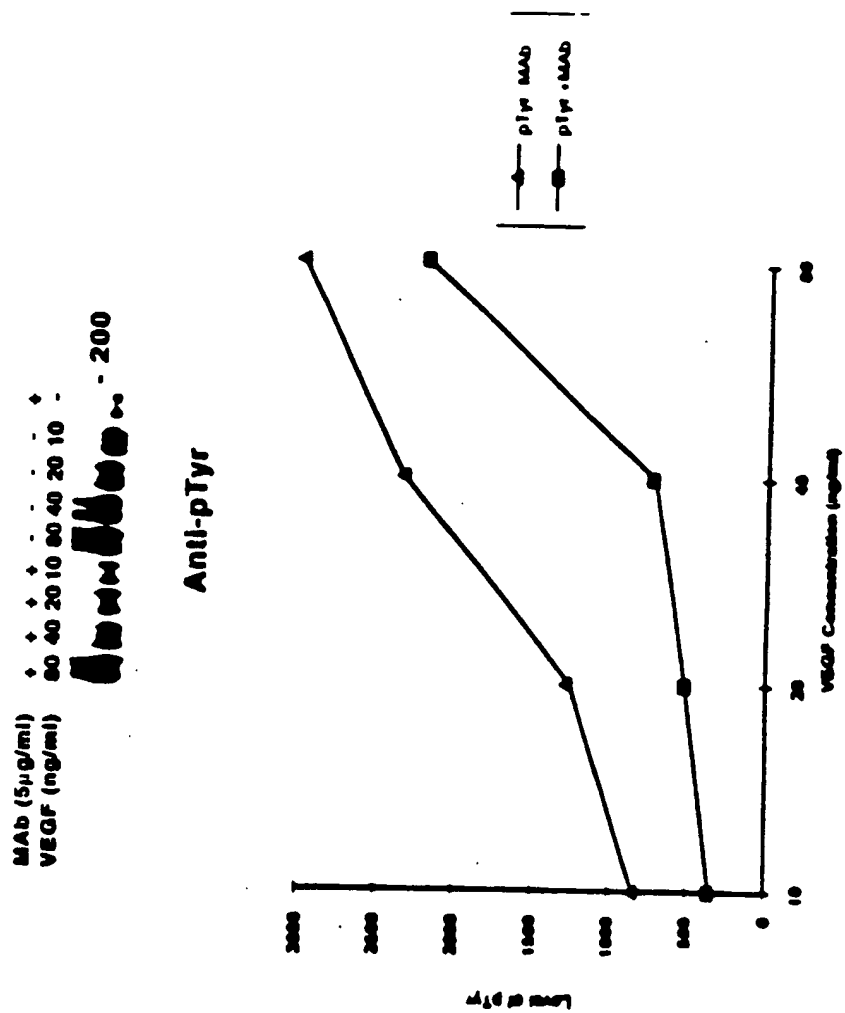
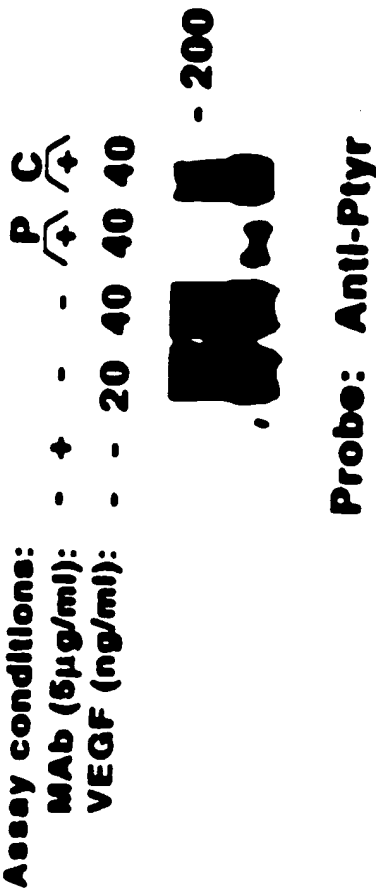


Figure 4

Inhibition of VEGF-Flk-1/ms activation by prebound MAb DC101



Assay conditions: P: MAb prebound 15'; VEGF 15'
C: Competitive assay: MAb + VEGF 15'

Figure 6

**FACS Analysis of Anti-flk-1 MAb Binding to flk-1/fms
Transfected 3T3 Cells (C441)**

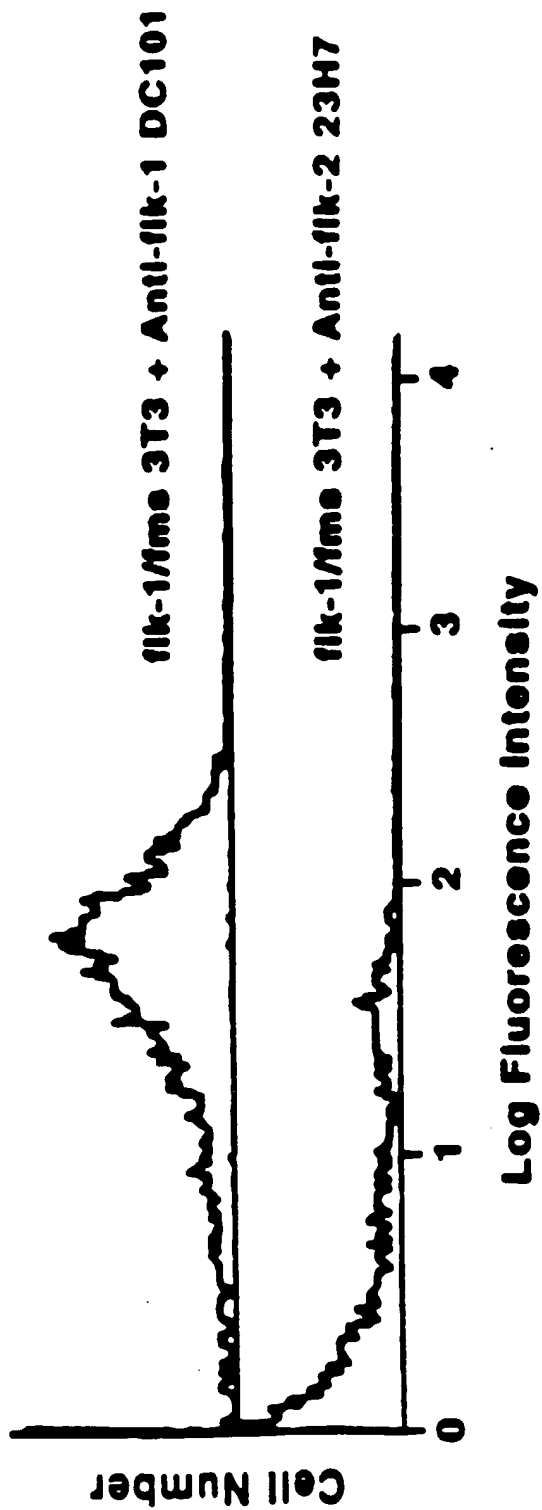


Figure 7

**Saturation Binding of MAb DC101 to the
Flk-1/fms Receptor on the Transfected 3T3 Cell Line C441**

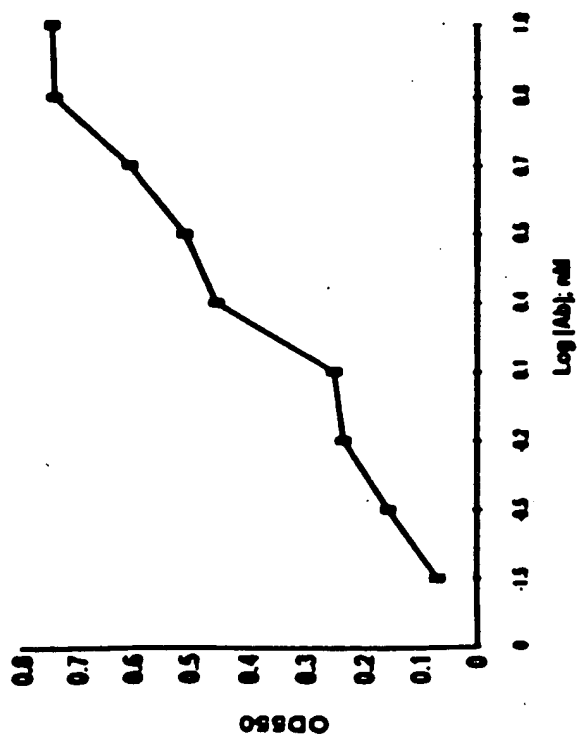


Figure 8

**Immunoprecipitation of phosphorylated flk-1/fms from
VEGF stimulated flk-1/fms transfected 3T3 cells.**

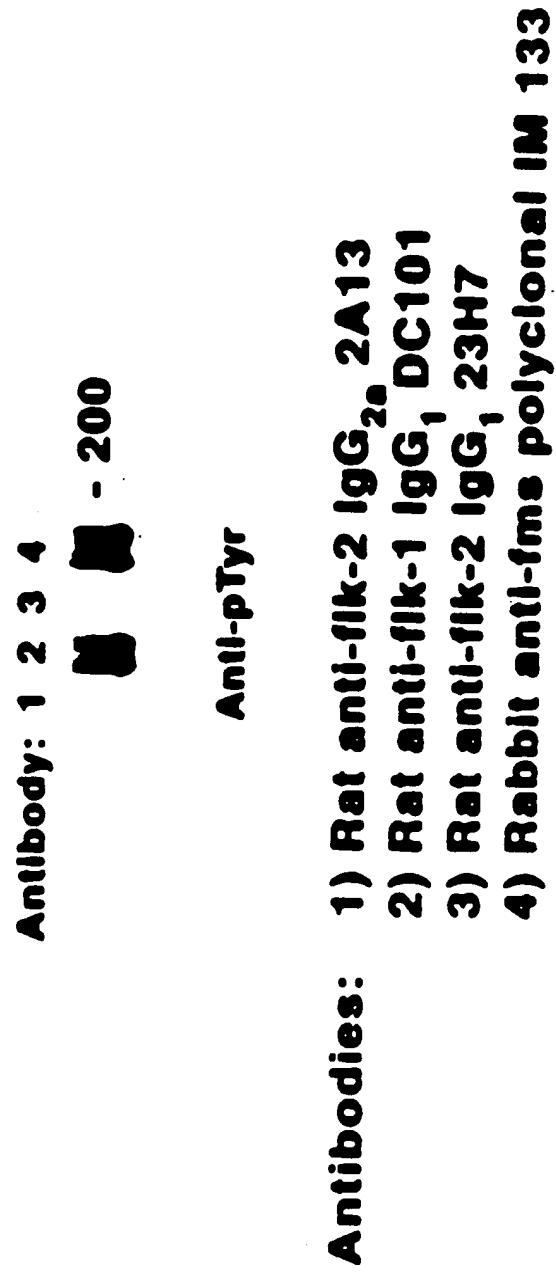


Figure 9

**Sensitivity of VEGF induced phosphorylation of the
Flk-1/fms receptor to inhibition by MAb DC101**

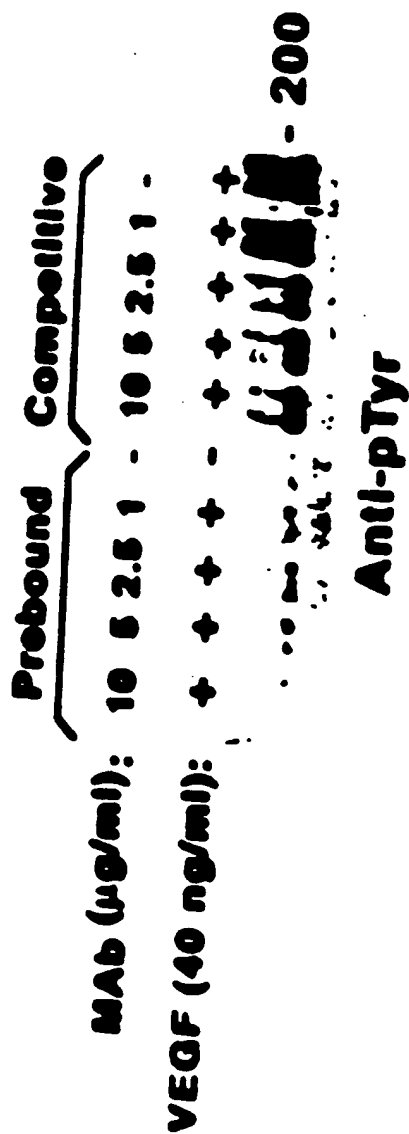


Figure 10

Effect of MAb DC101 on CSF-1 induced phosphorylation of the FMS receptor.

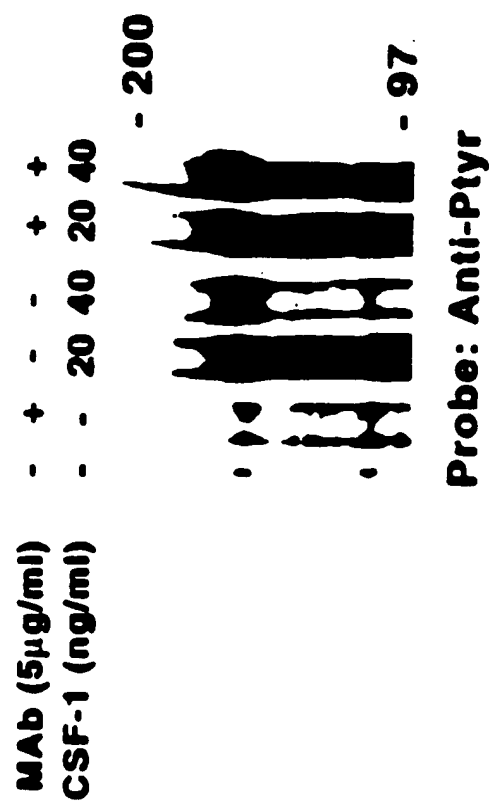


Figure 11

Specificity of MAb DC101 neutralization of the activated flk-1/fms receptor

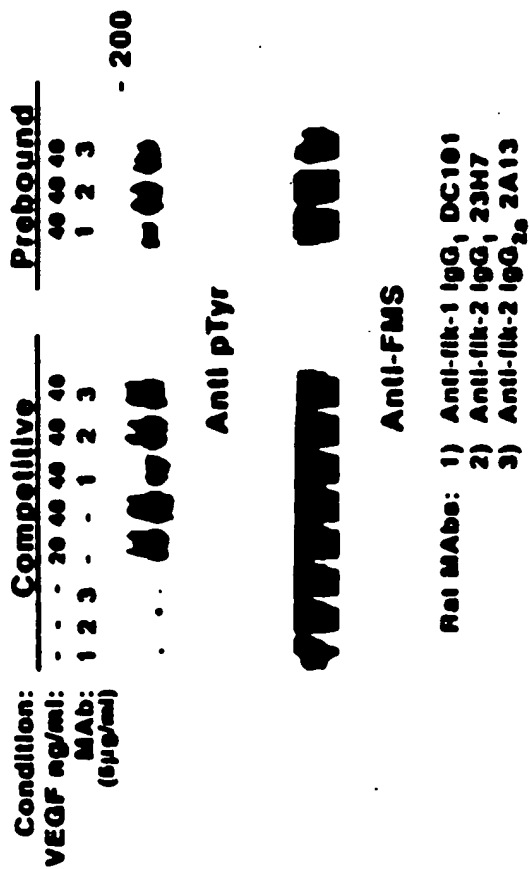
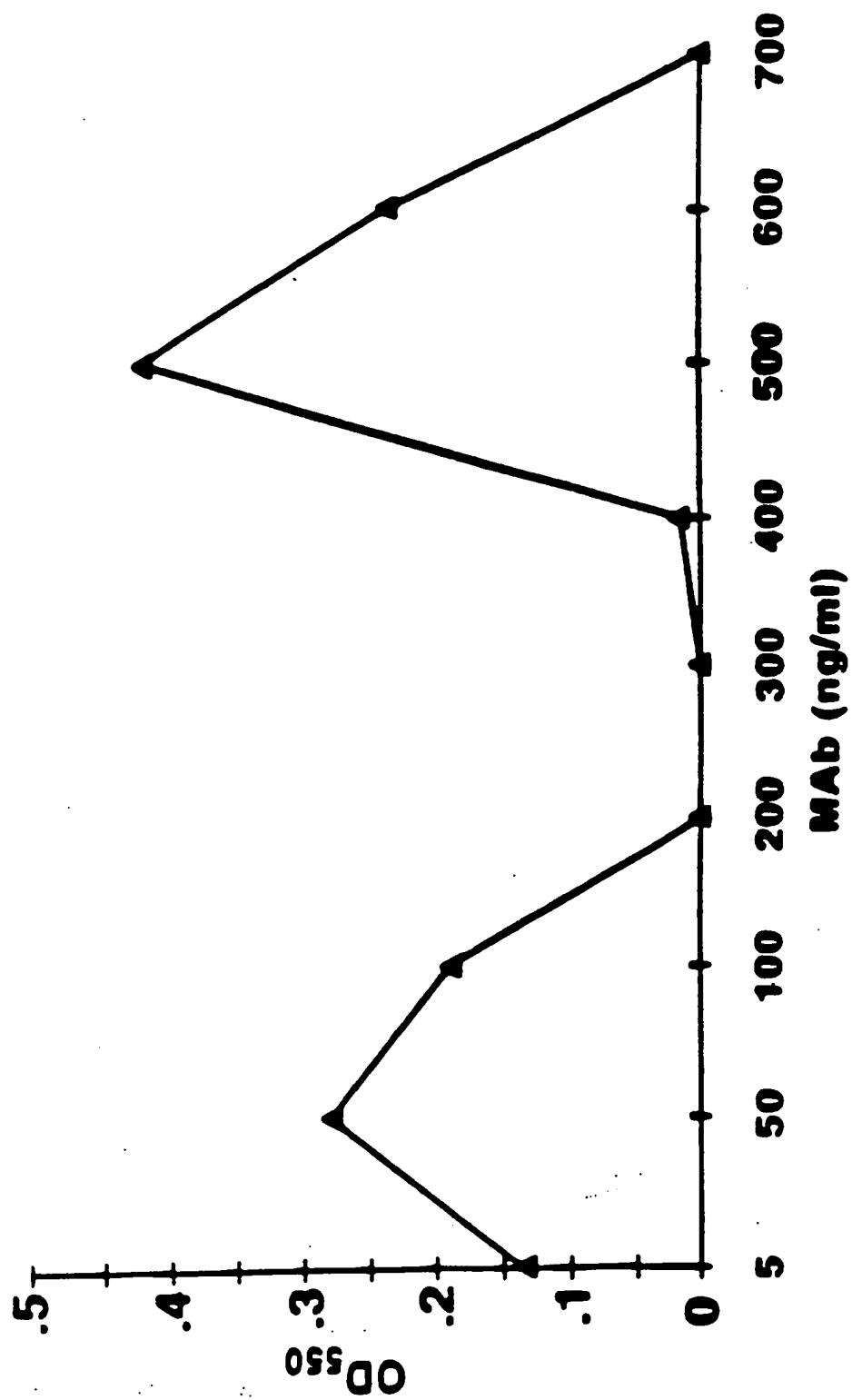


Figure 12



Immunoprecipitation of phosphorylated receptor bands from VEGF stimulated HUVEC cells

Figure 13

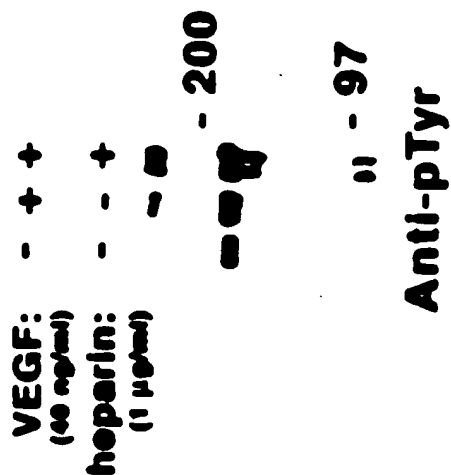


Figure 14

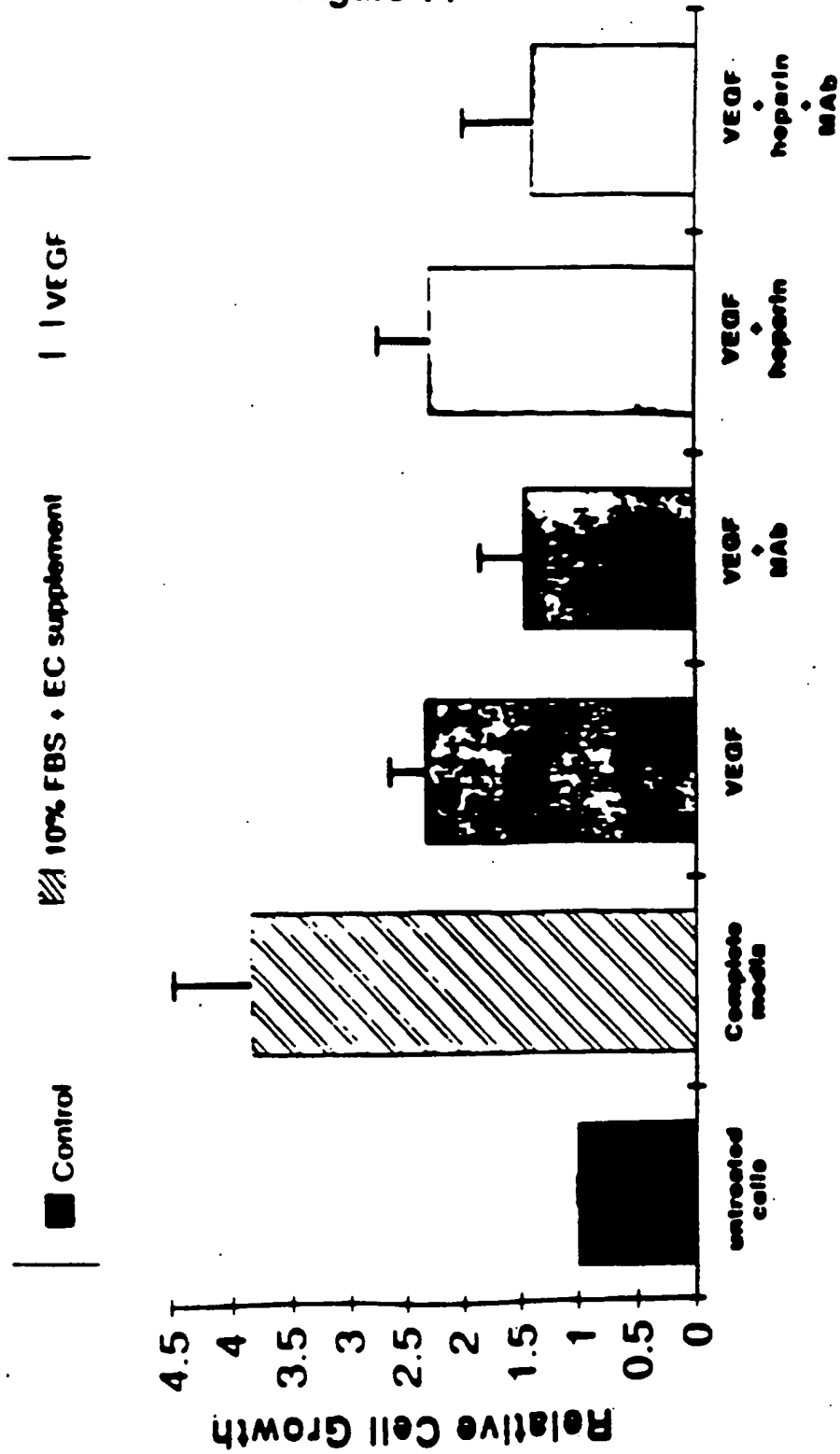


Figure 15

**Effect of MAb DC101 on VEGF Receptor Forms in the
Tumor Cell Lines A431 and 8161**

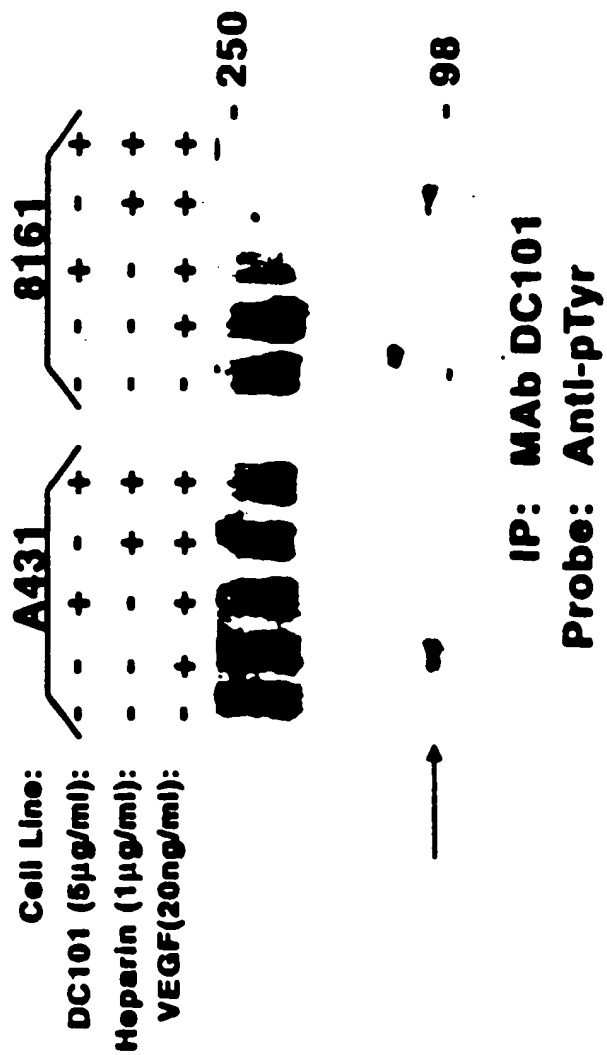
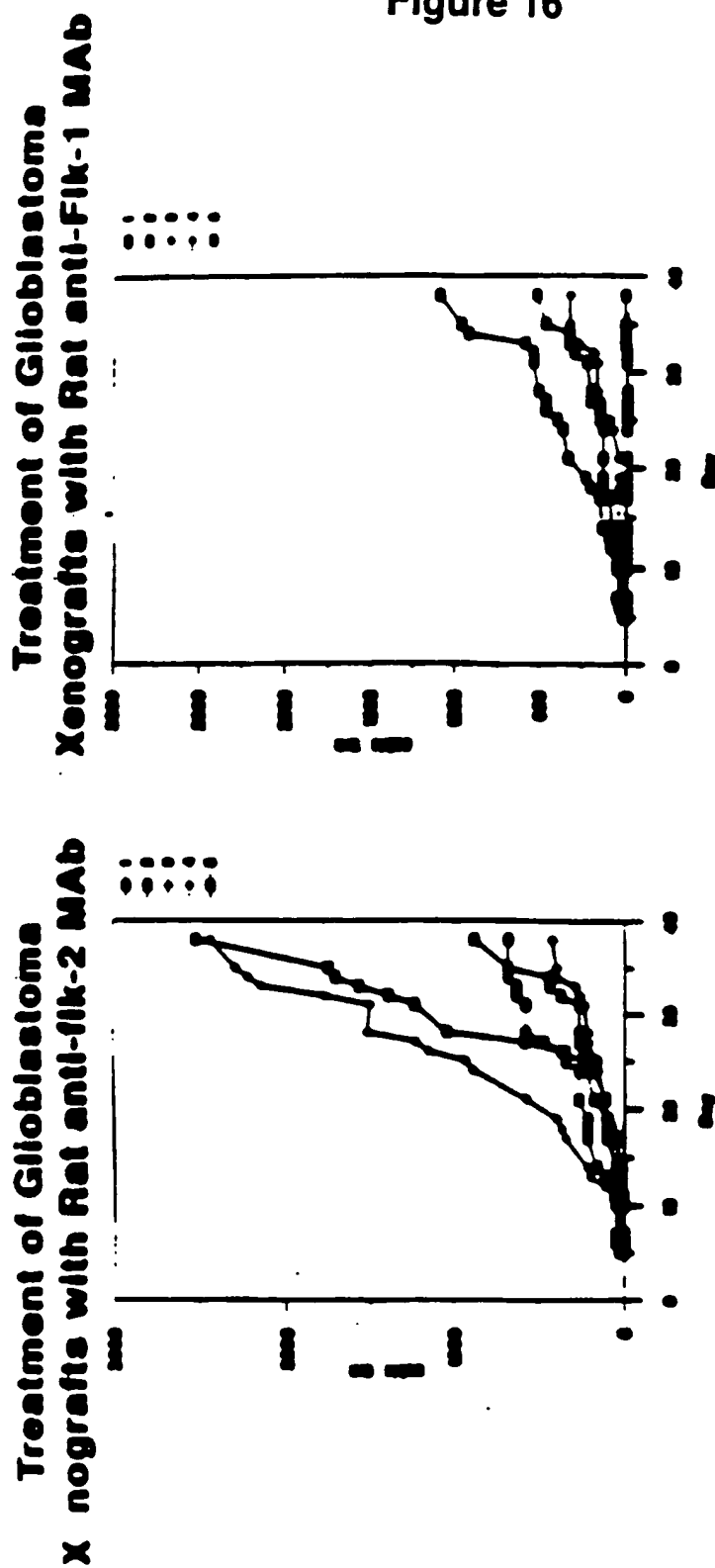


Figure 16

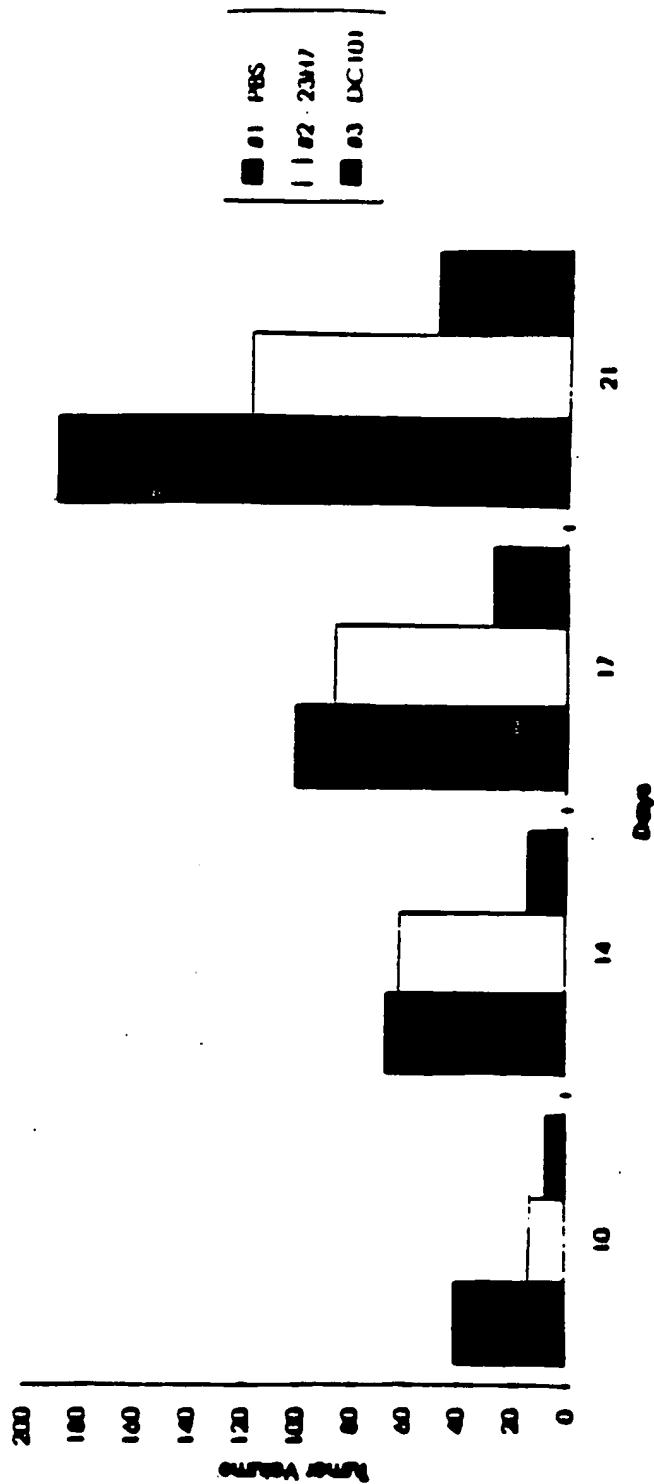
**Statistical Analysis:**

Flk-1 slope = 16.09

Flk-2 slope = 37.39

p value for Flk-1 versus Flk-2 tumor size = 0.0001

Figure 17



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01678

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 530/387.3, 388.22, 323; 435/240.27; 536/23.5; 424/133.1, 143.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3, 388.22, 323; 435/240.27; 536/23.5; 424/133.1, 143.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, DIALOG: EMBASE, MEDLINE, BIOSIS, WPI, BIOTECH ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 92/14748 (TERMAN ET AL) 03 SEPTEMBER 1992, SEE ENTIRE DOCUMENT.	1-29
Y	CELL, VOLUME 72, ISSUED 26 MARCH 1993, BIRGIT MILLAUER ET AL, "HIGH AFFINITY VEGF BINDING AND DEVELOPMENTAL EXPRESSION SUGGEST FLK-1 AS A MAJOR REGULATOR OF VASCULOGENESIS AND ANGIOGENESIS" PAGES 835-846, SEE ENTIRE DOCUMENT.	1-29
Y	JOURNAL OF EXPERIMENTAL MEDICINE, VOLUME 178, ISSUED DECEMBER 1993, ARJA KAIPAINEN ET AL., "THE RELATED FLT4, FLT1, AND KDR RECEPTOR TYROSINE KINASES SHOW DISTINCT EXPRESSION PATTERNS IN HUMAN FETAL ENDOTHELIAL CELLS" PAGES 2077-2088, SEE ENTIRE DOCUMENT.	1-29
Y	US, A, 5,270,458 (IHOR R. LEMISCHKA) 14 DECEMBER 1993	1-29

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be of particular relevance
* E		earlier document published on or after the international filing date
* L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	* A	document member of the same patent family

Date of the actual completion of the international search
10 APRIL 1995

Date of mailing of the international search report

04 MAY 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer
SUSAN LORING

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01678

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GROWTH FACTORS, VOLUME 7, ISSUED 1992, K. JIN KIM ET AL, "THE VASCULAR ENDOTHELIAL GROWTH FACTOR PROTEINS: IDENTIFICATION OF BIOLOGICALLY RELEVANT REGIONS BY NEUTRALIZING MONOCLONAL ANTIBODIES", PAGES 53-64, SEE ENTIRE DOCUMENT.	1-29
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCE, VOLUME 81, ISSUED NOVEMBER 1984, SHERIE L. MORRISON ET AL., "CHIMERIC HUMAN ANTIBODY MOLECULES: MOUSE ANTIGEN -BINDING DOMAINS WITH HUMAN CONSTANT REGIONS DOMAINS", PAGES 6851-6855, SEE ENTIRE DOCUMENT.	11-13
Y	NATURE, VOLUME 351, ISSUED 1991, MAN SUNG CO ET AL, "HUMANIZED ANTIBODIES FOR THERAPY", PAGES 501-502, SEE ENTIRE DOCUMENT.	16-18

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US95/01678

A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C07K 16/28, 16/18, 14/485; C12N 5/18; C07H 21/00; A61K 49/00, 51/10